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Identifying unique and overlapping roles of reactive oxygen species in rice blast and Southern corn leaf blight

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Plants and their fungal pathogens both produce reactive oxygen species (ROS). Cytotoxic ROS act both as stressors and signals in the plant-fungal interaction. In biotrophs, a compatible interaction generates little ROS, but is followed by disease. An incompatible interaction results in a strong oxidative burst by the host, limiting infection. Necrotrophs, in contrast, thrive on dead and dying cells in an oxidant-rich local environment. Rice blast, *Magnaporthe oryzae*, a hemibiotroph, occurs worldwide on rice and related hosts and can decimate enough rice each year to feed sixty million people. *Cochliobolus heterostrophus* , a necrotroph, causes Southern corn leaf blight (SLB), responsible for a major epidemic in the 1970s. The objectives of our study of ROS signaling and response in these two cereal pathogens were:

1. Confocal imaging of ROS production using genetically encoded redox sensor in two pathosystems over time.

2. Forward genetic screening of HyPer sensor lines in two pathosystems for fungal genes involved in altered ROS phenotypes.

3. RNA-seq for discovery of genes involved in ROS-related stress and signaling in two pathosystems.

Revisions to the research plan: Library construction in SLB was limited by low transformation efficiency, compounded by a protoplasting enzyme being unavailable during most of year 3. Thus Objective 2 for SLB re-focused to construction of sensor lines carrying deletion mutations in known or candidate genes involved in ROS response. Imaging on rice proved extremely challenging, so mutant screening and imaging were done with a barley-infecting line, already from the first year.

In this project, ROS imaging at unprecedented time and spatial resolution was achieved, using genetically-encoded ratio sensors in both pathogens. This technology is currently in use for a large library of rice blast mutants in the ROS sensor background, and Southern corn leaf blight mutants in final stages of construction. The imaging methods developed here to follow the redox state of plant pathogens in the host tissue should be applicable to fungal pathogens in general. Upon completion of mutant construction for SCLB we hope to achieve our goal of comparison between intracellular ROS status and response in hemibiotroph and necrotroph cereal pathogens.

Contribution of the collaboration:

Work in the two labs was integrated from the start of the project; we compared notes during the visit of the US co-PI Nicole Donofrio and graduate student Tim Chaya to the Horwitz lab in February 2018. In particular, we discussed imaging methods and screening strategies. Later, the Israeli PI was a member of Tim Chaya's thesis committee. The Technion lab received the optimized MoHyper construct from the U.D. lab and modified it for expression in *C. heterostrophus*, and provided M6-pHyper to the U.D. lab. Thus, from the technical point of view, for SLB, the collaboration was particularly important in the second year: the UD lab developed imaging methods for SLB following the methods for rice blast optimized in year 1 of the project. Tim Chaya transformed constructs from the Horwitz lab into SLB and the material was transferred back to the Israeli lab for analysis. From the scientific point of view, we have had numerous internet conference calls to discuss data and actively discussed further collaborations. Israeli coPI Benjamin Horwitz met with members of the UD lab at the GSA's fungal genetics conference in Asilomar, CA in March 2019, and visited at the Donofrio lab and UD Bioimaging center after the meeting.

Achievements

Confocal imaging of ROS over time in both pathosystems: After optimization of constructs and methods in both labs, lines expressing the ratiometric fluorescent H_2O_2 reporter Hyper were imaged in plant host tissue for both rice blast and SLB (Appendix, Figures 2, 4-6). A perfusion system was designed for real-time ratiometric imaging (Figure 4). Although not suitable for high-throughput screening, the system gives high-resolution kinetics for individual germinating spores and will be ideal for second-stage mutant screening. The results of imaging *in planta* (Figures 2 and 4) show that in rice blast, there is a peak in oxidation state when the fungal hyphae proliferate in the first invaded cell. Ratio analysis of images like the one shown in Figure SLB, on the other hand, showed a more uniform state with some variation in oxidation level between hyphal extensions, but no striking dependence (like the one in rice blast, Figure 4) of redox state on the z-coordinate of the confocal sections, which correspond to penetration into the leaf. For SLB this will need further time points. Overall, establishment of the ROS imaging system for both pathogens addressed the main goal of Objective 1.

Forward genetic screening of HyPer sensor lines in two pathosystems for fungal genes involved in altered ROS phenotypes: The Donofrio lab tested protocols to screen the library of 2,006 mutants generated in our *Magnaporthe oryzae* hyper line (MoHyPer). The perfusion system will facilitate the second-stage screening.

RNA-seq: For this we have been awaiting the results of mutant screening in rice blast and construction of mutants in SLB, to process a full set of samples, so this objective was not yet addressed in the time frame of the project. The new CEL-Seq based RNA-seq protocol at the Technion Genome Center has reduced the price of sequencing, and the RNA-Seq can be done at any time with additional funds available in the Horwitz lab.

Revisions to the research plan: Library construction in SLB was limited by low transformation efficiency, compounded by a protoplasting enzyme being unavailable during most of year 3. Thus Objective 2 for SLB re-focused to construction of sensor lines carrying deletion mutations in known or candidate genes involved in ROS response. Imaging on rice proved extremely challenging, so mutant screening and imaging were done with a barley-infecting line, already from the first year.

Publications for Project IS-5024-17 R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun

2. Appendix

Our findings have not yet reached publication stage; the data are reported in more detail here. We expect to communicate comparative ROS time/spatial imaging of wild type and mutants of the two pathogens shortly. Some additional funding is available in both labs to continue the experimental work.

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Summary of unpublished data

2.1. ROS reporter line, Southern corn leaf blight

2.1.1. Reporter construct

Reporter line M6-pHyper, in the C4 *tox+ MAT1-2* background, grows and sporulates normally and causes normal lesions on maize leaves (Figure 1). In year 1, the Technion lab received the MoHyper construct developed at the University of Delaware lab and tried unsuccessfully to transform *C. heterostrophus* (SLB). The promoters for the selection marker (Bialaphos resistance) and the MoHyper coding sequence are from *Magnaporthe oryzae* and might not drive sufficient expression in SLB. We therefore cloned the MoHyper coding sequence into a vector from BH lab. The selection marker is hygromycin B resistance, with the hygromycin phosphotransferase gene expressed under the Aspergillus promoter PTrpC, which has been tested in SLB. Likewise, MoHyper was cloned downstream of the Aspergillus Pgpd promoter, which has also been tested in SLB and gives strong constitutive expression. Three hygromycin-resistant transformants were isolated. Mycelia sampled after one day growth on complete medium amended with hygromycin were observed in the confocal microscope in complete medium with 10 mM H_2O_2 to optimize the long-wavelength excitation of MoHYper. No clear fluorescence stronger than the autofluorescence of WT controls was observed upon laser excitation at 488 nm. As a result, we returned to the original construct which was well expressed in SLB. We chose reporter line M6-

pHyper as our standard, and similar, independently isolated transformants also gave good ratio fluorescence signals in the imaging system at UD.

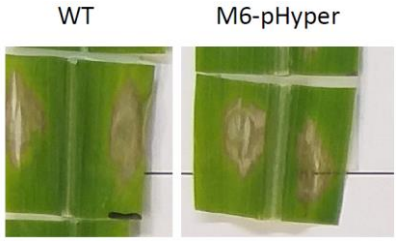

Figure 1. Lesions following infection of maize (cultivar Royalty) with WT C. heterostrophus or the M6-pHyper expressing line. Intact plants were infected with a 2x2x3 mm agar block with adhering mycelia, incubated 24 h in a sealed bag at 100% relative humidity, and grown for an additional 3 days at ambient humidity in a growth room following removal of the inoculum. (B.A.H. unpubl.).

With improved microscopy since the original imaging *in vitro* we were able to obtain good images of line M6-pHyper on and inside the maize leaf (Figure 2). The construct was transferred to the UD group (we cannot export SLB strains to the US), and Tim Chaya from the UD group transformed *C. heterostrophus* strain C4, so that after year 1 we had pHyper-expressing *C. heterostrophus* lines in both laboratories.

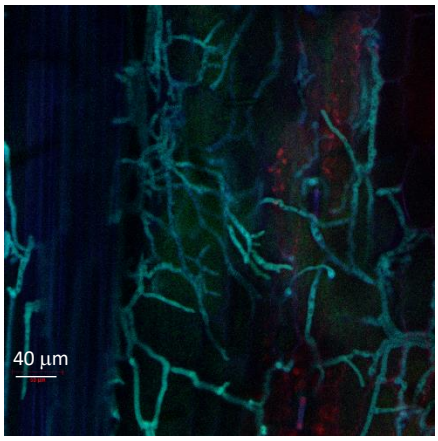

Figure 2. M6-pHyper sensor detects location-specific ROS levels during in-planta infective development. Left: M6-HyPer expressed in *C. heterostrophus* hyphae (fluorescence excitation at 405 and 488 nm) growing inside a maize leaf at 12hpi, between the epidermis and mesophyll cells (mesophyll cells contain red autofluorescent chloroplasts). Images were taken with a ZEISS LSM710 confocal. Image: Ariella Alperovitch-Lavy, Yael Lupu-Haber & Nitsan Dahan.

To determine the location of the insertion of the pHyper vector into the genome in M6-pHyper, we followed two strategies, genome sequencing and plasmid rescue. Plasmid rescue, by self- ligation of genomic DNA digested with restriction enzymes that do not cut within the plasmid backbone and ampicillin resistance marker of the construct, was unsuccessful (perhaps related to the complicated multicopy insertion, Figure 3). For the sequencing strategy, the Technion Genome Center used their newly-introduced Oxford Nanopore sequencing to sequence a M6-pHyper genomic DNA sample for us. On the second run, we obtained long, high-quality reads. Copies of the pHyper plasmid were found in a single contig of 54115 bp assembled from 30 read. Pairwise BLASTN alignments with the genes on the plasmid showed that pHyper was integrated in two tandem copies and three more partial copies (Figure 3A). Though it is always possible that additional copies were overlooked by the sequence assembly, it is clear that a multicopy insertion took place. The fungal sequence 3' to the insertions has an interesting property: it is also detected by BLASTN to the *C. heterostrophus* genome in multiple contigs (Figure 3B), suggesting that insertion occurred in a region of repetitive DNA. Two copies of a coding sequence with similarity to a reverse transcriptase gene found in other fungal genomces are located in this region, suggesting that it contains retrotransposon(s). These

data, maybe of interest for studies of the genome, which is known to contain retrotransposons (Santana et al. 2014, BMC Genomics doi: 10.1186/1471-2164-15-536). Of relevance here, insertion in such a region is less likely to perturb gene expression and physiology, supporting the use of M6-pHyper as a suitable line on which to perform additional mutagenesis to study redox-relevant genes.

Figure 3. M6-pHyper integration in C. heterostrophus. A)map showing multiple integration of the vector. The small diagram indicates the order and direction of the genes and expression signals on the plasmid; the diagram in color shows BLASTN alignments of each marker to the genomic sequence. B) BLASTN results showing best hits for the region with no homology to the plasmid (right-hand side in A) upon searching the C. heterostrophus genome at the JGI (https://mycocosm.jgi.doe.gov/CocheC5_3/CocheC5_3.home.html)

2.1.2. Mutant isolation/construction, *C. heterostrophus* (SLB)

At the time we started library construction by insertional mutagenesis, we were unable to obtain large quantities of Driselase needed for protoplasting, and also suffered from a low (2-3 isolates per transformation with 10 μg DNA). Our aim in the project was the isolation, by screening of a mutagenized pHyper-expressing line, of mutants of SLB with altered cytosolic basal redox state or altered response to ROS. With one year of the project remaining, we decided, instead, to construct mutants in candidate genes involved in redox metabolism (Table 2.1). As mentioned in the Year 2 report, we also planned to use our previous *chap1* pHyper strain, however it could not be revived from the freezer, and we are restarting construction of this one too. The constructs to generate these lines have been made and correct assembly verified by sequencing (Roni Koren & Ariella Alperovitch-Lavy); transformations into *C. heterostrophus* C4 are in progress (November 2020).

2.1.3. Redox imaging

The ratio protocol was set up at Technion by Nitsan Dahan at the LS&E microscopy center, together with Tim Chaya from the UD group, who was online with us while testing the imaging parameters. We obtained images similar to those shown in Figure 4B, recorded at UD.

2.2. Rice blast

2.2.1. Background on mutant screen

The Donofrio lab has generated 2,006 mutants in our *Magnaporthe oryzae* hyper line (MoHyPer). Originally, we were going to screen all mutants using the HyPer construct, however high-throughput screening has proved challenging (see below for details). Hence, all mutants have been screened singly in 140 µM menadione-containing media, nitrogen starved media and a complete media. Mutants with aberrant phenotypes are then processed through a secondary solid screen. In parallel with the mutagenesis screen, we tried to establish assays to screen these mutants rapidly and consistently. In the UD Bioimaging center, we have coupled a gravity perfusion system with a Zeiss LSM710 confocal microscope which has allowed for real-time kinetic analysis of both fungi responding to external stimuli. Figure 2A shows the response generated by MoHyPer to 10 mM hydrogen peroxide, which can be compared to Figure 2B showing M6-pHyPer under the same conditions. With this system in place it has allowed us to treat isolated conidia with different solutions and measure the internal redox state. This system will allow us to reliably analyze the mutants generated in a very controlled environment. While this system is not feasible for high-throughput screening, we can now take the mutants collected from the primary and secondary screen, and further analyze them for their ROS detection spectra. Due to Covid-related complications, we were unable to get to this stage at this time. While labs closed for about four months, the "lag time" was actually much longer, and because undergraduates were not allowed back in, and

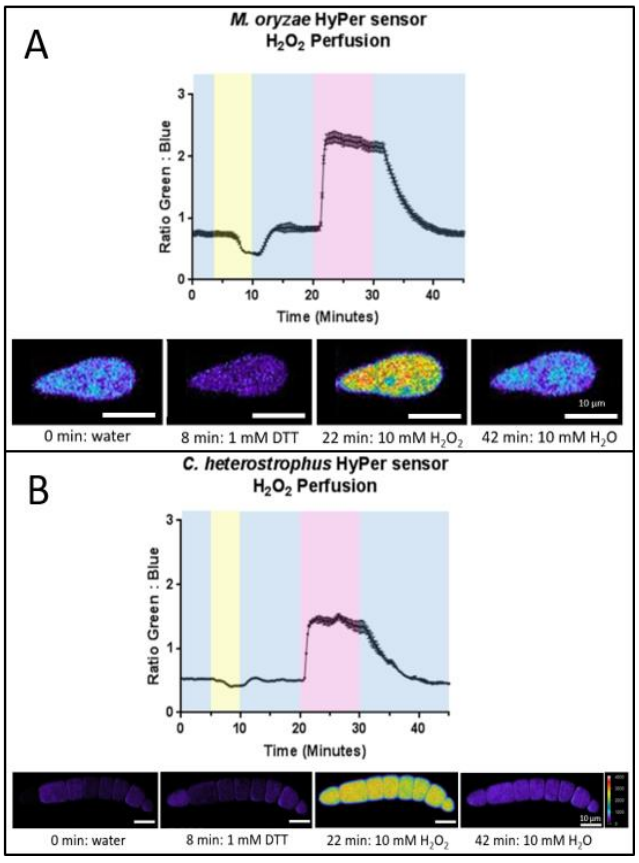

Figure 4. **A)** MoHyPer in a gravity perfusion system where 1 mM DTT and 10 mM H_2O_2 are flowed across isolated conidia. Below are selected images at 0, 8, 22 and 42 minutes of exposure. Blue = low, Red = high redox levels. **B)** *C. heterostrophus* M6-pHyPer in the same assay.

we were unable to access equipment such as the BioImaging center, due to capacity, it was more like a seven-month lag time, and we are still feeling its effects.

2.2.2. Comparative ROS and growth habits in *C. heterostrophus* and *M. oryzae*

In addition to conducting *in vitro* assays, we have made progress on analyzing the redox states of *M. oryzae* and *C. heterostrophus* during host infection (Objective 1). Our MoHyPer line was inoculated onto barley cv. Lacey and imaged through the early stages of infection. The goal of this was to ascertain a point where select future mutants could be screened. Between 2 and 24 hours, we could not detect a consistent or significant change in ROS levels. However, we are continuing to assay between 36-48 hours which will encompass appressorial formation, penetration and expansion in the first epidermal cell. We have also imaged at 36- and 48-hours post inoculation and there appears to be spatial differences between the levels of MoHyPer in barley related to interactions with the epidermal cell walls (Figure 4). It will be important to quantify this difference related to the spatial locations of the invasive hyphae within the host.

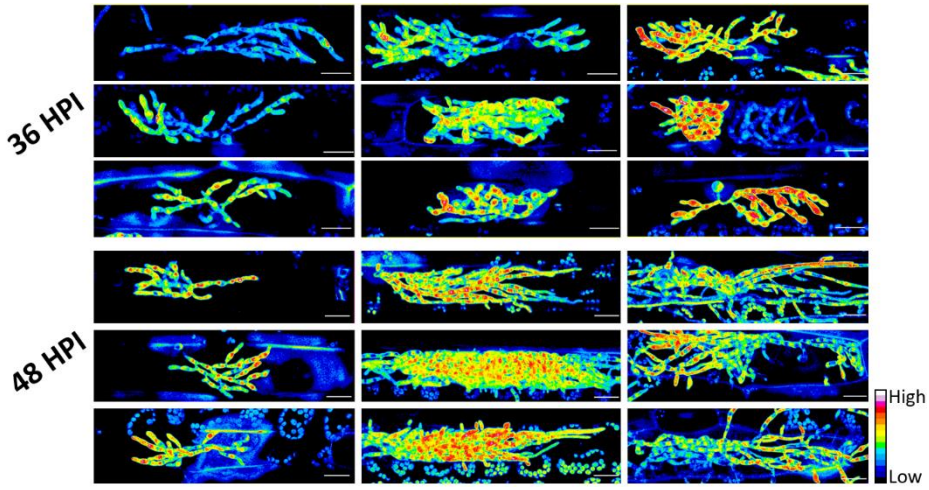

Figure 5. MoHyPer infection of barley cv. Lacey at 36 and 48 hours post inoculation (HPI). Blue = low Red = high oxidative levels. At the 36HPI time point more red is seen within the hyphae and as infection progresses to 48 hours, the ROS detection decreases.

Advances have been made for live imaging of *C. heterostrophus* infection progression on a maize leaf. *C. heterostrophus*-pHyPer was imaged on B73, a highly studied maize line, starting at 45 minutes through 9 hours post inoculation. B73 leaves were inoculated and imaged every 15 minutes for 9 hours. The image series was then analyzed in FIJI and cropped if the sample went out of focus, or fluorescence decreased below detection level. Heat maps were generated where each spore cell was cropped along with the tip of the extending germ-tube, and if formed, the appressoria. Figure 5 shows 3 heat maps of different outcomes of inoculation – No infection, single appressoria formation and penetration, and dual appressoria formation. The figures show the spores as the cropped cells and then germ tubes and appressoria are GT and A with numbers to differentiate in the image analysis. What is notable is the tip cells have increased level of ROS during germination, and then if an appressorium is formed, there is a feedback that is seen to the spore. Data has been acquired and is being analyzed of *C. heterostrophus* HyPer inoculated on cellophane

to investigate if there is a change in ROS level in the absence of a plant host defense response. Future efforts need to be made to see if these increases change during a resistant host infection.

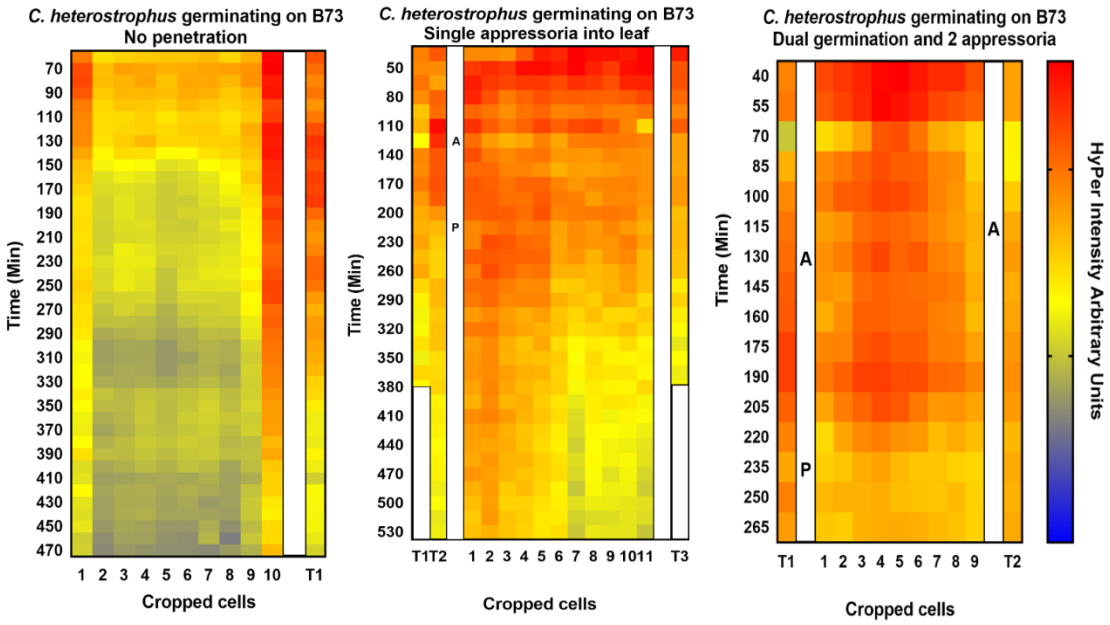

Figure 6: Data from FIJI was generated and used to generate Heat maps in GraphPad Prism. Spores germinated on B73 leaves for at least 30 minutes before imaging on a Zeiss LSM710 confocal microscope. Each column represents a cropped spore cell, the tip of a germ tube, and the germinating tips (T) are numbered. On the heatmap A and P indicate Appressoria and Penetration respectively. The HyPer intensity is a measurement of the 488:405 ratio. Red = High. Blue = Low.

Our current goal is to complete the secondary screen for our mutants generated in MoHyPer and complete fluorescent analysis in select mutants with via the perfusion system as well as an assay for ROS levels *in planta* via H2DCF-DA. For *C. heterostrophus*-pHyPer we will complete *in planta* analysis in B73 to understand the dynamic changes during infection, and how they compare to rice blast.